CLONING AND EXPRESSION PROFILING OF DISCUS FISH (*Symphysodon aequifasciata*) PROLACTIN RECEPTOR (PRLR)

by

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LIST OF ABBREVIATIONS

aa	amino acid
AMV	Avian Myeloblastosis Virus
ANOVA	analysis of variance
BLAST	basic local alignment search tool
$CaCl_2$	calcium chloride
cDNA	complementary deoxyribonucleic acid
CIP	calf intestinal phosphatase
CRH	cytokine receptor homology
DEPC	diethylpyrocarbonate
dfPRLR	discus fish prolactin receptor
dH_2O	distilled water
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DT	DNase treatment
dNTP	deoxyribonucleoside triphosphate
EC	electrochemical
ECD	extracellular domain
EDTA	ethylenediaminetetraacetic acid
EPO	erythropoietin
FP	forward primer
FNP	forward nested primer
GC	guanine-cytosine
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPTG	isopropyl β-D-thiogalactopyranoside
JAK	Janus kinase
LB	Luria-Bertani

MAPK	mitogen-activated protein kinase
$MgCl_2$	magnesium chloride
M-MLV	Moloney murine leukemia virus
MOPS	4-morpholinepropanesulfonic acid
mRNA	messenger RNA
MS	mammosomatotroph
NaCl	sodium chloride
NCBI	National Center for Biotechnology Information
ORF	open reading frame
PCR	polymerase chain reaction
PL	Placental lactogen
PRL	Prolactin
PRLR	Prolactin receptor
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
RNase	ribonuclease
RP	Reverse primer
RNP	Reverse nested primer
RT	reverse transcriptase
RT-PCR	reverse transcription-polymerase chain reaction
SEM	standard error of the mean
SL	somatolactin
STAT	Signal Transducers and Activators of Transcription
TAP	tobacco acid pyrophosphatase
TPO	thrombopoietin
UTR	untranslated region
UV	Ultraviolet
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

PENGKLONAN DAN PENGPROFILAN PENGEKPRESAN RESEPTOR PROLAKTIN (PRLR) DARIPADA IKAN DISKUS (*Symphysodon aequifasciata*)

ABSTRAK

Ikan diskus (*Symphysodon aequifasciata*) ialah sejenis ikan hiasan yang terkenal dengan sifat penjagaan keibubapaan yang berterusan. Ikan diskus induk menyara anak-anaknya dengan rembesan mukus badan yang penting untuk tumbesaran dan perkembangan larva yang berenang bebas. Prolaktin (PRL) sudah lama menunjukkan kesan secara langsung yang mempengaruhi sifat penjagaan keibubapaan dalam pelbagai spesies haiwan vertebrata. cDNA PRLR ikan diskus (dfPRLR) yang lengkap telah diklonkan. dfPRLR didapati mengandungi 2214 bp yang mengekodkan protein matang sepanjang 632 asid amino. Jujukan asid amino dfPRLR mengandungi peptida isyarat andaian sepanjang 23 asid amino, domain ekstrasel 210 asid amino, domain transmembran tunggal 24 asid amino dan domain intrasel 375 asid amino. Pelbagai ciri superfamili reseptor sitokin kelas 1 telah dicamkan dalam klon ini termasuk dua pasangan residu sisteina dan satu motif WS dalam domain ekstrasel, satu domain transmembran tunggal, rantau Petak 1 dan Petak 2 dalam domain intrasel. Jujukan asid amino dfPRLR yang disimpulkan ini mempunyai kesamaan identiti sebanyak 34 ke 79% dengan ikan dan 29 ke 36% dengan vertebrata. Keputusan pengekspresan tisu dewasa pula menunjukkan pengekspresan dfPRLR adalah tertinggi dalam organ-organ osmoregulasi termasuk buah ginjal, perut, insang dan usus kecil, diikuti pengekspresan yang sederhana dalam otak dan ovari. Hati, otot dan kulit menunjukkan pengekspresan dfPRLR yang rendah. Selain itu, kajian perbandingan PRLR kulit dalam ikan diskus induk dan bukan-induk menunjukkan kenaikan yang signifikan dalam pengekspresan mRNA PRLR bagi kedua-dua ikan induk jantan dan betina. Keputusan ini membuktikan dfPRLR terlibat dalam mekanisme osmoregulasi dan fungsi pembiakan. Fungsi dfPRLR yang mengawalatur sifat penjagaan keibubapaan dalam pelbagai aktiviti sel epidermis seperti pembezaan sel mukosa, perkembangan dan penghasilan mukus turut dibincangkan.

CLONING AND EXPRESSION PROFILING OF DISCUS FISH (*Symphysodon aequifasciata*) PROLACTIN RECEPTOR (PRLR)

ABSTRACT

Discus fish (*Symphysodon aequifasciata*) is a popular ornamental fish that uniquely exhibits extensive parental care behaviour. Parental discus fish feed their newborns with bodily-secreted mucus which is essential for the growth and development of free swimming larvae. Prolactin (PRL) has long been shown to directly influence parental care associated behaviour in many vertebrate species. We cloned the full-length cDNA of discus fish PRLR (dfPRLR). dfPRLR consists of 2214 bp, encoding a 632 amino acid mature protein. The deduced amino acid sequence of dfPRLR composes a putative signal peptide of 23 amino acids, an extracellular domain of 210 amino acids, a single transmembrane domain of 24 amino acids and an intracellular domain of 375 amino acids. Several characteristics of the Class 1 cytokine receptor superfamily were identified in this clone including two pairs of cysteine residues and a WS motif in the extracellular domain, a single transmembrane domain, Box 1 and Box 2 regions in the intracellular domain. The deduced amino acid sequence of dfPRLR shared 34 to 79% identity among fish and 29 to 36% with vertebrates. Adult tissue expression results depicted significantly higher expression of dfPRLR in the osmoregulatory organs including kidney, stomach, gill and intestine followed by an appreciable level of expression in brain and ovary. Liver, muscle and skin showed low expression of dfPRLR. Moreover, comparative studies of skin PRLR in parental and non-parental discus fish showed a significant elevation in PRLR mRNA expression in both male and female parental fish as compared to non-parental fish. Our findings support the fact that dfPRLR is involved in osmoregulatory mechanisms and also reproductive functions. The possible roles of dfPRLR that regulate its parental care behaviour in various epidermal cell activities such as mucosal cell differentiation, proliferation and production of mucus are discussed.

CHAPTER 1

INTRODUCTION

1.1 General

Over the past few decades, extensive use of molecular biology and genetic engineering techniques that help enhance the reproduction as well as increase the productivity of freshwater fish such as salmon, trout, catfish, goldfish and carp have been carried out successfully. The above-mentioned techniques have also helped develop disease resistance among freshwater fish. At the economy level, freshwater fish represent a global multi-million-dollar industry, therefore their importance to local as well as international economy cannot be overlooked. Biological experimentation in fish contributes significantly to an overall increase in the understanding of the molecular biology, gene cloning, gene sequencing, gene expression and regulation of fish. In a broader context, the results will also unquestionably enhance work in other fields, such as aquatic-based nutraceuticals, food industry as well as microbiology (Dunham *et al*., 2000).

Discus fish belong to a large family of fish, Cichlidae. It was first introduced in the 1920's and is now regarded as one of the most beautiful of all ornamental fish. They are elegant and colorful with high market value. Nevertheless, their popularity is constantly rising seeing that it is one of the most discussed fish species even until these days. Proudly, Malaysia is one of the largest exporters of discus fish and other freshwater tropical aquarium fish worldwide. The popularity of the discus has given it its nickname among aquarists: the King (Koh *et al*., 1999).

The importance of prolactin (PRL) is evident from the wide spectrum of functions it performs in vertebrates and its degree of conservation throughout the evolution process. The isolation of homologous PRLs and prolactin receptor (PRLRs) from a variety of fish species will allow us to understand better the evolution of the PRL/GH/SL gene family, the role of PRL in osmoregulation, and physiological mechanisms on targeted tissues and organs. The complexity of PRL's actions is mirrored by the complexity of factors involved in regulating

PRL synthesis and secretion and the interactions of PRL with other endocrine systems (Manzon, 2002).

Much of recent work has focused on the isolation and characterization of piscine PRLs and their receptors. It became apparent that molecular biological approaches would be needed in order to achieve a better understanding of the actual mechanisms and pathways governed by fish PRLRs. A more widespread approach involving more animal models is therefore essential. To our knowledge, this approach has not been used in discus fish. Thus, this project will be a very promising pioneer study in fish endocrinology.

This project is designed in line with the research effort of our fish laboratory. Lately, we have discovered certain protein composition in discus fish epidermal mucus (Chong *et al*., 2005a), the relationship between mucus and fry development and the role of mucus in discus physiology and parental behaviour. PRL and PRLR are important factors in regulation of mucus secretion and parental behaviour, therefore this project can further clarify the mechanisms and hormonal regulations in mucus secretion of discus fish, hence complement the whole research effort of our team.

1.2 Objectives

In this project, we seek to clone a novel PRLR homolog from a freshwater cichlid, the discus fish. This unique species displays the ability to regulate epidermal mucus secretion, which we believe is governed by PRL and PRLR. Hence, we opt to study the structure and function of discus PRLR. Successful cloning of the discus PRLR gene can enable us to characterize the transcriptional activities of the discus PRLR gene in different tissues and organs under different conditions. This project will hopefully shed new insights on the role of PRL in freshwater teleosts in particular and higher vertebrates in general.

In short, the objectives of this research project are as follows:

- To clone and characterize the full-length cDNA of discus PRLR
- To study the expression of discus PRLR in different tissues
- To compare the expression of discus PRLR in non-parental and parental phase

CHAPTER 2

LITERATURE REVIEW

2.1 Prolactin (PRL)

Back in 1920s, little was known about the anterior pituitary. Studies on anterior pituitary extracts showed that they contained some substances which could induce growth and stimulate reproductive organs (Smith, 2004). In 1928, Stricker and Grueter from France discovered an anterior pituitary factor which was able to stimulate developed mammary gland to secrete milk (Manzon, 2002). This discovery was confirmed and extended to other vertebrates soon after. A couple of years later, this anterior pituitary factor was identified as a hormone and named prolactin (PRL) after lactation as it was a lactation stimulating factor in pigeon crop sac (Riddle *et al*., 1933).

Gradually, studies showed that the effects of PRL weren't merely to stimulate reproductive organs, growth and milk secretion by the mammary gland. Researchers were trying to identify the broad functions of PRL but at that time they were hampered by limited techniques available. The discovery of radioimmunoassay techniques initiated extensive studies of PRL in mammals, avian and even in fish species (Susan Smith, 2004). To date, more than 300 biological effects were identified for this multifaceted hormone (Bole-Feysot *et al*., 1998).

Studies of PRL in fish began only in 1950s (Manzon, 2002). During that period, bioassays were mostly done on euryhaline species by using heterologous PRL. Injection with purified PRL on hypophysectomized killifish enabled them to survive in freshwater environment (Pickford & Philips, 1959). This successful research induced intensive studies on body fluid balance in other euryhaline species including stickleback (*Gillichthyes mirabilis*), molly (*Poecilia latipinna*) and tilapia (both *Oreochromis niloticus* and *Oreochromis mossambicus*) (Horseman, 1987 in Manzon, 2002). For other teleosts, effects of PRL in osmoregulation may vary among different species. Later, research in molecular and functional disciplines has been carried out to further verify the diverse functions of PRL.

Results have clearly verified that PRL acts on osmoregulatory organs including gill, kidney, intestine and urinary bladder and regulates the ion transport mechanisms in different fish species (Manzon, 2002).

2.1.1 Structure

Along with the development of cloning technology, sequence, structure, binding, distribution and evolution of PRL were gradually elucidated and the studies have clearly shown that PRL is a polypeptide hormone secreted from anterior lobe of the pituitary and belongs to the protein hormone family same as growth hormone (GH), mammalian placental lactogen (PL) and teleostean somatolactin (SL) due to various structural similarities (Bole-Feysot *et al*., 1998; Wallis, 1981; Wallis, 2000). From the evolutionary studies, GH, PRL, PL and SL came from a common ancestral gene and underwent divergence independently. GH and PRL evolved almost in a similar rate and pattern hence they shared relatively high homology in the structure and function (Wallis, 2000).

The 3D crystallographic structure of human PRL (hPRL) (Figure 2.1) shows that it is an all-alpha-helix protein containing 4 bundles of alpha-helix very similar to GH. In a molecular context, hPRL is located on chromosome 6 containing 5 exons and 4 introns. hPRL is composed of 914 nucleotides with 681 nucleotides of ORF encoding a prehormone of 227 amino acids, 28 aa as a signal peptide and 199 aa of mature hormone, with a total molecular mass of about 23kDa. All PRLs identified thus far are about 200 aa containing six cysteines forming three intramolecular disulfide bonds (Cooke *et al*., 1981; Bole-Feysot *et al*., 1998).

Fish PRL is obviously shorter compared to mammalian PRL (Figure 2.2). Fish PRL is located on chromosome 3 containing 5 exons and 4 introns. Fish PRL is synthesized as a prohormone of approximately 200 amino acids, 23-24 aa as a signal peptide and 161-165 aa of mature hormone, with a total molecular mass of about 19kDa (Ensor, 1978). Teleostean PRL lacks one disulfide bridge if compared with the mammalian and non-teleostean PRL because teleostean PRL lacks about a dozen residues at the N terminal

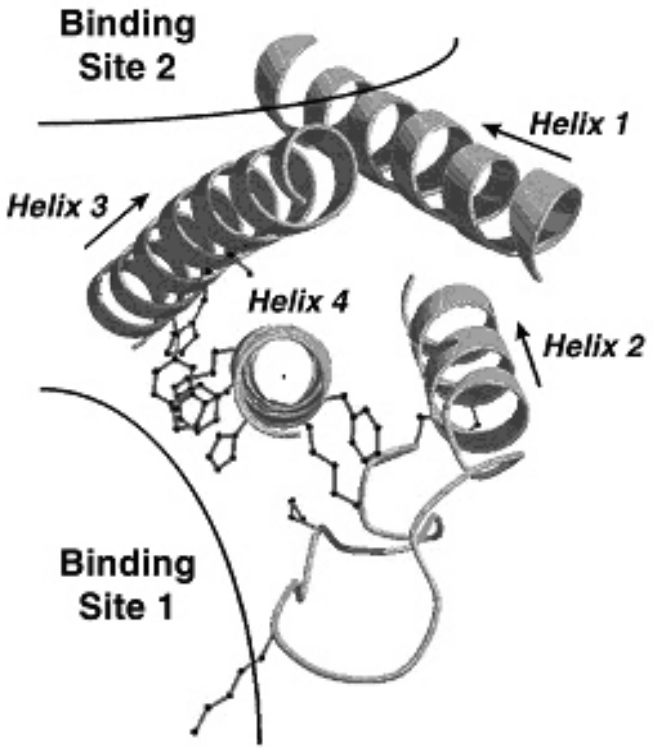
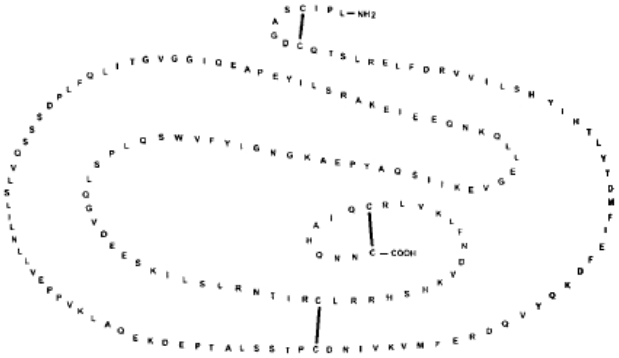
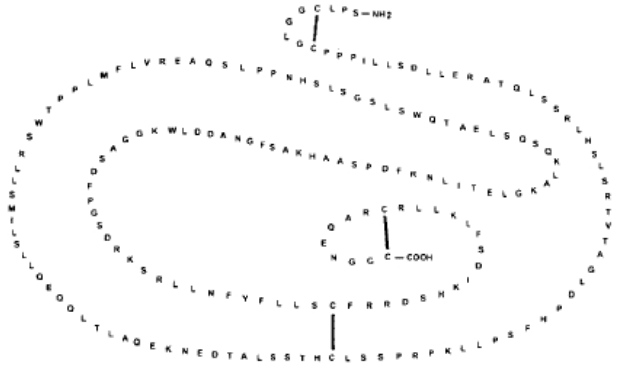


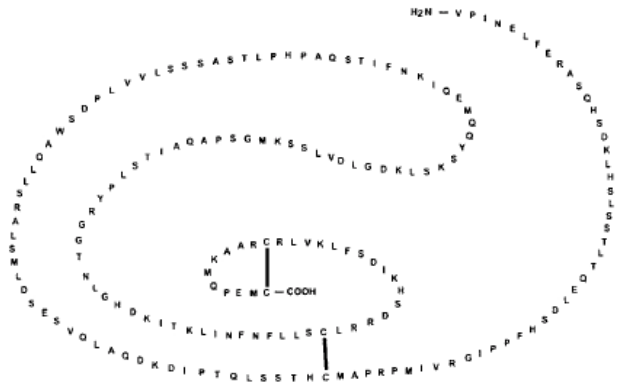
Figure 2.1 Ribbon representation of the predicted 3D structure of hPRL, modeled on the basis of the crystallographic structure of porcine GH. hPRL is predicted to adopt the four-helix bundle folding described for GHs (adopted from Figure 1 A; page 227; Bole-Feysot *et al*., 1998).



mouse PRL



sturgeon PRL



Nile tilapia PRL_{188}

Figure 2.2 Structural comparison of fish PRLs with mammalian (mouse) PRL. Teleostean PRLs (i.e., Nile tilapia PRL_{188}) lack the N-terminal disulfide bridge which is found in mouse PRL and appear to be more similar in structure to mouse GH. Conversely, nonteleostean fish like the sturgeon have retained the N-terminal bridge and more closely resemble mouse PRL. References for amino acid sequences presented in this figure: mouse PRL, Harigaya *et al.,* 1986; sturgeon PRL, Noso *et al.,* 1993; Nile tilapia PRL_{188}, Rentier-Delrue *et al.,* 1989. (adopted from Figure 1; page 293; Manzon, 2002)

(Rand-Weaver *et al*., 1993 in Bole-Feysot *et al*., 1998). The lacking of N-terminal disulfide bond may suggest the principle role of PRL in osmoregulation in teleost species (Manzon, 2002) and tilapia PRL has no activity on mammary gland or pigeon crop sac (Ensor, 1978). This suggestion was supported by the studies on disruption of the N-terminal disulfide bond of ovine PRL. The disruption was found to increase the potency in teleost osmoregulation bioassay (Doneen *et al*., 1979).

The survival of fish always depends on aquatic environment and this requires many physiological regulations which are very different from the terrestrial life. Among them, body fluid regulation is of particular importance as the body are exposed to media of changing salinities. Therefore homeostatic control of body fluid balance is crucial in fish species. To this reason, some osmoregulatory hormones of fish should have undergone functional and molecular evolution during the ecological transition to the terrestrial life (Takei *et al*., 2006). This explains very well the existence of multiple forms of PRL and the wide spectrum of PRL functions (Specker *et al*., 1985; Yasuda *et al*., 1986, 1987; Chao *et al*., 1988; Yamaguchi *et al*., 1988; Rentier-Delrue *et al*., 1989). The evolution of PRL also suggests the increased efficiency and specificity of its actions to permit the colonization of freshwater habitats (Manzon, 2002).

Despite the diverse functions, the structure of PRL shows some conserved domains across the vertebrate taxa. Conserved domains were suggested to play some vital roles in ligand binding for the transmembrane receptor or the soluble forms of receptors found in the plasma (Manzon, 2002).

2.1.2 Production and distribution

In mammals, the distribution of PRL covers a wide range of tissues. Primarily, PRL is synthesized in the lactotroph or mammotroph of the anterior pituitary gland. Also, there is an intermediate cell population known as mammosomatotrophs (MS) which is able to produce PRL as well as GH. In the presence of estrogen, MS is able to differentiate into lactotrophs (Freeman *et al*., 2000). Apart from the anterior pituitary gland, PRL is also

produced by numerous other cells and tissues including various regions of the brain, decidua, myometrium, lacrimal gland, thymus, spleen, circulating lymphocytes, and lymphoid cells of bone marrow, mammary epithelial cells and tumors, skin fibroblasts, and sweat glands. Studies showed that extrapituitary PRL compensates pituitary PRL in certain unforeseen circumstances (Bole-Feysot *et al*., 1998).

In fish, PRL is found to be secreted primarily from rostral pars distalis of the pituitary. There were also evidences to show that PRL is produced in proximal pars distalis and the pars intermedia of teleost species (Huang and Specker, 1994). The existence of MS cells in fish hasn't been fully studied. MS cells were first observed in gilthead sea bream and appeared to be pluripotent in the ontogenetic study of gilthead sea bream (Villaplana *et al*., 2000). This suggests that the cells can differentiate into either GH or PRL cells depending on their location and the physiological needs of the organisms. In some other fish species, extrapituitary PRL has been observed using immunocytochemical bioassays or *in situ* hybridization in brain, liver, intestine, gonad, kidney, spleen, gill and muscle (Santos *et al*., 1999; Imaoka *et al*., 2000; Lee *et al*., 2006a).

2.1.3 Stimulation and regulation

PRL regulates its effects via a series of actions. Pituitary PRL generally acts through a classic endocrine pathway by secreting PRL into the circulatory system and transported to the target cells through specific binding to the corresponding transmembrane receptor namely PRL receptor to initiate the signal transduction mechanisms (Bole-Feysot *et al*., 1998; Freeman *et al*., 2000). To date, no specific studies on PRL stimulation and regulation in fish have been done but the effects of piscine PRL are typically shown to be mediated by a receptor (Manzon, 2002).

In other vertebrates, PRL is found to bind with plasma PRL-binding proteins in order to generate a fast response to sudden physiological changes. PRL-binding proteins can also serve as a protection to PRL to avoid degradation of circulating the PRL (Kline & Clevenger, 2001; Manzon, 2002). Both external and internal stimulations regulate the

secretion of PRL in vertebrates. In mammals, the most prominent physiological stimulations that elevate pituitary PRL secretion are suckling, stress, and increased levels of ovarian steroids, primarily estrogen (Neill et al., 1974 in Freeman *et al*., 2000).

Besides, lactotrophs regulate PRL secretion through various releasing factors (autocrine regulation) or by other cells within the pituitary gland (paracrine regulation) (Freeman *et al*., 2000). Extrapituitary PRL also can act in a more direct way either in an autocrine or paracrine manner. As an autocrine, PRL binds to receptor on the same cell for further effects and as a paracrine, locally produced PRL can act on adjacent cells. Using these paracrine or autocrine mechanisms, it is possible for PRL to act without interfering the concentration of the circulating hormone (Bole-Feysot *et al*., 1998).

2.2 Prolatin Receptor (PRLR)

PRL initiates its effects by binding to its receptor, PRLR. Binding of PRL to its receptor will induce a signal transduction mechanism and hence regulate the cellular responses and physiological functions of the organisms. PRLR belongs to hematopoietin receptor superfamily that is pertaining to the class 1 cytokine receptor superfamily that includes receptors for GH, erythropoietin (EPO), thrombopoietin (TPO), leptin and the interleukins (Drachman & Kaushansky, 1995; Bole-Feysot *et al*., 1998; Kelso, 2000). PRLR is a single-pass, transmembrane chain comprising of three major domains; extracellular, transmembrane and intracellular domains. Several features in these domains are highly conserved and this facilitates the classification of the receptor.

2.2.1 Cytokine receptor family

Cytokine is a very diverse class of signaling proteins and glycoproteins that involve in cellular communication. Hence, a cytokine receptor is a receptor which mostly appears on a plasma membrane that binds the cytokine to show effects (Ibelgaufts, 2007). The ability of cytokines to induce cell growth and differentiation depends on their recognition and binding to specific receptors. Cytokine and its receptor form specific binding complex in order to

transduce the binding of messenger cytokine into cytoplasmic signals that trigger physiological and developmental processes within the cell (Bazan, 1990).

In recent classification studies, receptors with notable amino acid homology and conservation of sequence motif characteristics have allowed these receptors to be grouped into a gene/receptor family. In the cytokine receptor family, receptors were classified based on details of their 3D structural establishment (Ibelgaufts, 2007). The largest group of cytokine is the hematopoietic factors formed by 50 different lymphokines, GH, hemopoietins, neuropoietins and interleukins (Drachman & Kaushansky, 1995; Kelso, 2000). Despite low sequence homology, these hematopoietic cytokines share a common four-alpha-helix protein fold topology. This conserved protein-protein interaction mode is capable of granting high specificity ligand binding (Drachman & Kaushansky, 1995; Foster *et al*., 2004). Divergence in sequence has given rise to differences in structure, thus allowing the classification of hematopoietic cytokines into three main types; short chain, long chain and interferon-like cytokines. Further investigation split these three types of cytokines into Class 1 (short and long chains) and Class 2 (interferon-like) cytokines (Mui & Miyajima, 1994; Kelso, 2000). Class 1 cytokine receptors have an intrinsic protein tyrosine kinase activity deficit while Class 2 cytokine receptors which mainly consist of interferons are multimeric composed of homologous subunits. PRLR is categorized under Class 1 (long chain) cytokine receptor superfamily which funnels its signal through conserved JAK/STAT intracellular pathways (Boulay *et al*., 2003).

2.2.2 Structure

Unlike hPRL, hPRL receptor is located on chromosome 5, containing at least 10 exons. Resulting from alternative splicing of the primary transcript, mammalian PRLR appears in multiple isoforms known as short, intermediate and long PRLR with respect to the length of the cytoplasmic tail of the receptor (Figure 2.3). The presence of the isoforms of PRLR coincides with the multiple forms of its hormone (Bole-Feysot *et al*., 1998). Fish PRLR is a

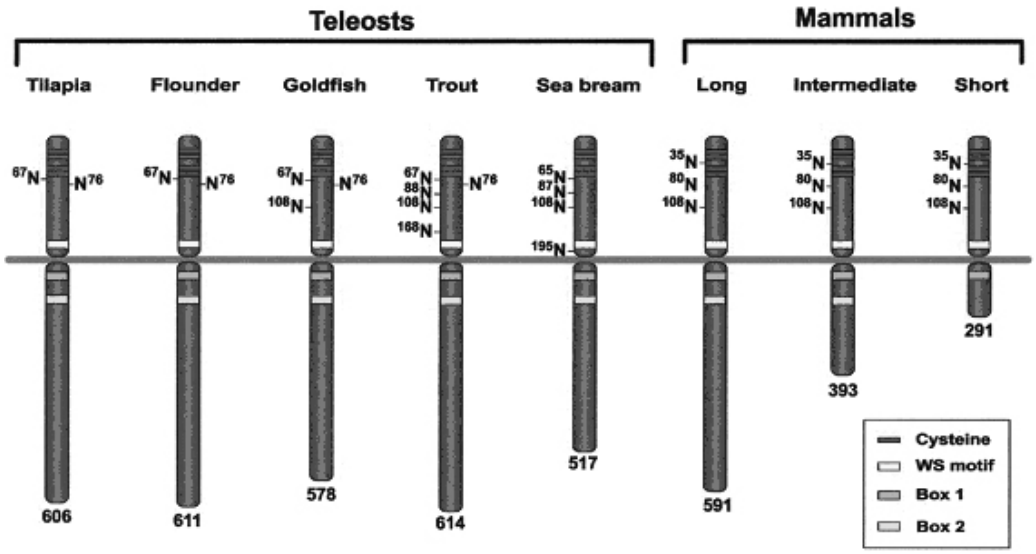


Figure 2.3 Comparison of fish and mammalian (rat) PRLRs. Receptor lengths are indicated below the individual receptors. The three mammalian isoforms have identical extracellular domains and differ in the length of their intracellular domains and signal transduction abilities. Sequence references: Nile tilapia, Sandra *et al.*, 1995; goldfish, Tse *et al.*, 2000; trout, Le Rouzic *et al.*, 2001; sea bream, Santos *et al.*, 2001; rat long, Shirota *et al.*, 1990; rat intermediate, Ali *et al.*, 1991; rat short, Boutin *et al.*, 1988 (adopted from Figure 2; page 297; Manzon, 2002).

mature protein of about 600 amino acids and in accordance to the long form of mammalian PRLR (Figure 2.3). The amino acid identity between fish and other vertebrates is around 30% (Manzon, 2002).

Most of the sequence similarities among cytokine receptors are found within their extracellular domain (ECD). Regardless the isoforms, PRLR ECD consists of about 210 amino acids in vertebrates. Two highly conserved features are found in this ECD including two pairs of disulfide-linked cysteines at the N-terminal and a pentapeptide termed "WS motif" (WSXWS, X = any amino acid) found in the membrane-proximal region of the C-terminal (Bole-Feysot *et al*., 1998; Goffin *et al*., 1998). For avian species, PRLR ECD is atypical. In pigeon and chicken, results showed that PRLR ECD is duplicated and contains two highly homologous Cytokine Receptor Homology (CRH) regions. This duplication of CRH does not seem to play any functional role since its deletion has no significant effect on the ligand-binding affinity, ligand specificity or signal transduction of pigeon PRLR (Gao *et al*., 1996; Chen & Horseman, 1994; Goffin *et al*., 1998).

Like all other cytokine receptors, PRLR is a single-pass transmembrane chain. The transmembrane domain of vertebrates is about 24 aa in length. To date, the involvement of this domain or of any crucial amino acid within this domain in the functional activity of the receptor has yet been explored (Bole-Feysot *et al*., 1998).

Intracellular or cytoplasmic domain is the least conserved domain among the three. The only well conserved region in this domain is the 8 amino acid sequence namely Box 1. It is a membrane-proximal region which is highly enriched in prolines and hydrophobic residues. Due to the unique structural properties of proline residues, the conserved P-x-P (x = any amino acid) motif within box 1 is suggested to correlate with the consensus folding and to recognize the transducer molecules (Bole-Feysot *et al*., 1998; Goffin *et al*., 1998). A less conserved region, Box 2 is not found in the short form of PRLR. Intracellular domain doesn't possess intrinsic enzyme activity but is related to the signal transduction mechanism. Box 1 involves in the activation of STAT or MAPK signal transduction pathways and this

suggests that signal transduction mechanisms in fish and mammals may have been conserved since Box 1 is very well conserved between fish and mammals (Freeman *et al*., 2000).

2.2.3 Distribution

The distribution of PRL-binding sites or receptors has been studied extensively over the past few decades. *In situ* hybridization and immunohistochemical studies have strongly proved that PRL-binding sites or receptors are widely distributed throughout vertebrates even in developing fetus (Royster *et al*., 1995; Freeman *et al*., 2000).

From the tissue distribution studies in fish species, PRLR shows high expression mainly in kidney, gill and intestine (Sandra *et al*., 1995, 2000; Le Rouzic *et al*., 2001; Prunet *et al*., 2000; Tse *et al*., 2000; Higashimoto *et al*., 2001; Santos *et al*., 2001; Lee *et al*., 2006a). This supports the primary role of PRL on osmoregulation in fish. Besides, PRLR expression was also observed in brain, gonad, liver, muscle, skin, spleen, head kidney, lymphocytes and bone of various fish species (Sandra *et al*., 1995, 2000; Tse *et al*., 2000; Higashimoto *et al*., 2001; Santos *et al*., 2001; Lee *et al*., 2006a). The wide distribution of fish PRLR in different tissues claims the co-evolution of PRL and its receptor throughout the evolutionary process among fish and mammals. This lends further support for the multifunctional characteristics of PRL. Cellular localization has been done to study in depth the exact sites of PRLR via *in situ* hybridization in those osmoregulatory organs namely chloride cells in gills, proximal convoluted tubule cells in kidney and mucosal epithelial cells in intestine (Manzon, 2002; Lee *et al*., 2006b).

2.2.4 Activation

Not only is the structure of PRLR well conserved between fish and mammals, the mechanism of ligand binding and receptor activation is also conserved. Activation of the PRLR is sequential and it involves a ligand-induced and receptor dimerization (Figure 2.4). Firstly, PRL hormone interacts with one molecule of PRLR through its binding site 1 (Figure 2.1) to form an inactive 1:1 complex. Formation of this complex appears to be a prerequisite

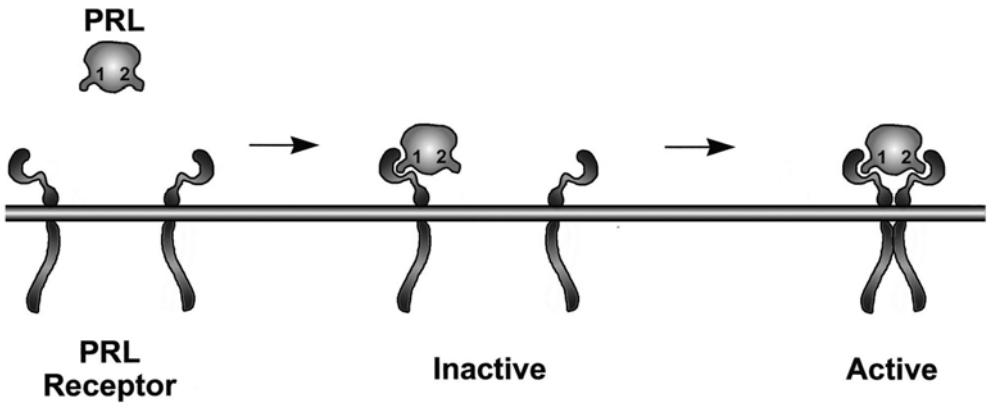


Figure 2.4 Mechanism of activation of PRLR by PRL-induced sequential dimerization. Firstly, the hormone (H) interacts with the receptor (R) through its site 1 to form an inactive H1:R1 complex. Secondly, the hormone binds to a second receptor through its site 2, which leads to receptor homodimerization and formation of an active trimeric H1:R2 complex (adopted from Figure 1 C; page 227; Bole-Feysot *et al.*, 1998).

for further action. Secondly, the hormone binds to a second yet identical molecule of the receptor through its binding site 2 to form an active 1:2 trimeric complex which composed of one hormone molecule and one receptor homodimer (Goffin *et al*., 1998; Bole-Feysot *et al*., 1998; Freeman *et al*., 2000; Manzon, 2002).

Dimerization of PRLR induces signal transduction pathway to initiate a cellular response in various target cells. PRLR itself doesn't possess any intrinsic enzymatic activity therefore tyrosine phosphorylation and activation of the JAK kinase followed by phosphorylation of the receptor are essential to start the signal transduction mechanisms (Rui *et al*., 1992; Taga & Kishimoto, 1993; Ihle, 1994; Kishimoto *et al*., 1994; Ihle & Kerr, 1995; Bole-Feysot *et al*., 1998). Besides JAK, another major signaling pathway involves the phosphorylation of cytoplasmic Stat proteins, which themselves dimerize and translocate to the nucleus and bind to specific promoter elements on PRL-responsive genes (DaSilva *et al*., 1994; Bole-Feysot *et al*., 1998).

Engineered hormone analogues with binding site 2 blocked sterically are unable to induce receptor homodimerization and receptors remain inactive. However, these analogues are still able to bind to binding site 1 of the receptor and hence act as antagonists to the wild type hormones (Goffin *et al*., 1998). Studies on mammalian (Gertler *et al.,* 1996) and trout (Sandowski *et al*., 2000; Le Rouzic *et al.,* 2001) PRLRs have revealed that the 1:2 complex is transient and rapidly dissociates to an inactive 1:1 complex. This is because an increase in the stability of the 1:2 complex may not guarantee a greater physiological response, whereas a rapid dissociation of the active trimeric complex back to the inactive form would give way to further functional interactions (Gertler *et al.,* 1996).

To support this argument, study on GH analogues showed that even if there was an increase in the affinity to the GH receptor, the analogues failed to enhance the signal transduction or cell proliferation (Pearce *et al.,* 1999). Another study suggested that there may be a "minimal time" for homodimering of the receptor in order to activate the signal transduction pathways (Helman *et al*., 2001).

2.3 Biological effects of Prolactin (PRL)

The significance of PRL studies lies in its broad area of biological effects in vertebrates. PRL was initially identified as a hormone from anterior pituitary which can stimulate the development of mammary glands (Riddle *et al*., 1933). Continuously, various other functions from this multifaceted hormone have been discovered and they have been reviewed and grouped into five broad areas including reproduction, osmoregulation, growth, integument and synergism with steroids (Nicoll & Bern, 1972; Nicoll, 1974 in Bole-Feysot *et al*., 1998). The number of effects of PRL kept on increasing and was then categorized into six categories; water and electrolyte balance, growth and development, endocrinology and metabolism, brain and behaviour, reproduction, and lastly immunoregulation and protection (Bole-Feysot *et al*., 1998).

All the actions mentioned in the cited references covered a wide range of animals including lower species and some of them may not directly be related to fish or mammals. This suggests that some actions of PRL may have been lost with evolution or may only be seen in higher animals during certain stages of development (Bole-Feysot *et al*., 1998). However, the present study is primarily on discus fish (*Symphysodon aequifasciata*) regarding its natural parenting behaviour and mucus production as a nourishment for young, hence other PRL effects will be described in Section 2.3.1 in general following the categories done by Bole-Feysot *et al*. (1998).

2.3.1 PRL effects in general

2.3.1.1 Water and electrolyte balance

From all the studies that have been done, PRL plays a key role in regulating fluid balance through the gill and kidney of many fish and has been referred to as a fresh water-adapting hormone (Sandra *et al*., 1995, 2000; Le Rouzic *et al*., 2001; Prunet *et al*., 2000; Tse *et al*., 2000; Higashimoto *et al*., 2001; Santos *et al*., 2001; Manzon, 2002; Lee *et al*., 2006a; San Martín *et al*., 2007). Water and electrolyte balance is of particular importance for fish as they are exposed constantly to media with varying osmotic degrees. Therefore, PRL plays

pivotal role in the homeostatic control of body fluid balance in freshwater and seawater fish (Bole-Feysot *et al*., 1998; Freeman *et al*., 2000; Manzon, 2002).

Seawater fish lose quite an amount of water to the hyperosmotic environment. In order to replenish the lost, seawater fish reabsorb the water from intestine and produce only a minimal volume of urine (Loretz, 2001). In freshwater, fish body fluids are hypertonic to the external environment. Freshwater fish are capable to reduce the gill permeability to retain the ions and salts while actively secrete excessive intake of water (Nicoll, 1974 in Bole-Feysot *et al*., 1998). For fish that migrate between fresh and seawater habitat, osmoregulation of water and electrolyte balance is highly essential (Bole-Feysot *et al*., 1998). Besides, there are also suggestions that PRL may protect the hatchlings of rainbow trout from failure in freshwater adaptation (Ensor, 1978).

2.3.1.2 Growth and development

There are a great amount of studies reporting that PRL stimulates growth and development. For some mammals and fish species, studies failed to prove that there is a direct effect of PRL on body growth but in birds PRL-treated males showed successful increase of body weight (Silverin, 1980). Since PRL and GH share high homology in amino acid and structural similarity, both of these hormones are suggested to share many biological functions in many vertebrates especially in the context of growth. In amphibians and reptiles, PRL reverses metamorphic changes but promotes skin molting (Tata *et al*., 1991; Bres & Nicoll, 1993; Bole-Feysot *et al*., 1998; Hasunuma *et al*., 2004). Antimetamorphic effect also occurs in fish species fundamentally in Japanese flounder (De Jesus *et al*., 1994). Regarding other reported studies on developmental processes, PRL also plays an important role in maturation of the lung and surfactant production, differentiation of preadipocytes, maturation of germ cells and hypothalamic dopamine development (Bole-Feysot *et al*., 1998). PRL is also found to affect angiogenesis (Corbacho *et al*., 2002).

2.3.1.3 Endocrinology and metabolism

In monkey, studies showed that PRL has specific effect on ATPase activity in different regions of the brain therefore modulating energy metabolism (Kumaran *et al*., 1989). PRL also has proven to regulate lipid and carbohydrate metabolism in mammals (Machida *et al*., 1990). In an endocrinology context, PRL was shown to have direct effect on pancreatic function and insulin secretion (Nielsen, 1982; Sorenson *et al*., 1987). Studies revealed that PRLR expression can be up- and down-regulated by GH and PRL depending on the concentration. Due to the wide spectrum of biological effects of PRL and the existence of multiple forms of PRLR, there is always more than one responsive mechanism designated for this multifaceted hormone. In order to respond efficiently to numerous target tissues, PRL is capable of acting promptly by increasing the number of specific PRLRs themselves (Kelly *et al*., 1991; Matsuda & Mori, 1997; Leclerc *et al*., 2007).

2.3.1.4 Reproduction

PRL has long been related to luteotropic and luteolytic actions and has been labelled as luteal hormone. In general, PRL enhances progesterone secretion by luteal cells. Results have strongly proven that PRL is involved directly in modulating the physiological states of oestrus, pregnancy, and lactation seeing the fact that it is an important factor in regulating the formation and destruction of the corpus luteum (Matsuyama *et al*., 1990; Cecim *et al*., 1995, Bole-Feysot *et al*., 1998; Freeman *et al*., 2000). In uterus, PRL increases the concentration of progesterone receptors and hence all uterine actions associated with progesterone are enhanced (Daniel *et al*., 1984; Chilton & Daniel, 1987). For male species, PRL has been proposed to stimulate steroidogenesis and androgen production when synergized with LH (Binart *et al*., 2003). PRL also increases total lipids and the conversion of spermatogenesis in Sertoli cells (Nag *et al*., 1981; Guillaumot & Benahmed, 1999). However, PRL has been reported that it causes a decrease in the gonads and the reproductive tract tissue weights in both male and female ring doves (Buntin & Tesch, 1985).

2.3.1.5 Immunoregulation and protection

Injection of PRL into hypophysectomized rats causes an increase in the weight of the spleen and thymus and promotes the thymus development (Carreño *et al*., 2004). In lymphocytes, PRL is known to increase hormonal and cellular immunity, to reverse anemia, leukopenia and thrombocytopenia that were induced by hypophysectomy. PRL is also found to enhance antibody formation, including IgG and IgM antibodies (Bole-Feysot *et al*., 1998; Freeman *et al*., 2000). In fish, PRL regulates melanogenesis (Sage, 1970) and maintains specific immune functions through prevention of immunosuppression by cortisol (Yada *et al*., 2004). To date, no genetic diseases associated with a mutation of the gene encoding PRL or the PRLR have been identified in humans or animals (Kelly *et al*., 1991). However, PRL has been associated with a number of different forms of cancer and tumor growth (Barker *et al*., 1992; Wennbo & Törnell, 2000). PRL is also shown to increase and to affect a number of autoimmune circumstances (Bole-Feysot *et al*., 1998).

2.3.2 Parental behaviour

Besides the well known effects of PRL in stimulating the production of milk proteins and osmoregulation, there is a wealth of evidence to support the idea that PRL is involved in parental behaviour of fish, birds, and mammals (Bole-Feysot *et al*., 1998; Schradin & Anzenberger, 1999; Freeman *et al*., 2000). In behavioural studies, parental care is a good example that would seem to benefit the species. It promotes the survival and well-being of the next generation at a cost to the resources of the current generation in order to ensure the continuity of the species (Freeman *et al*., 2000; Gross, 2005). This link between PRL and parental care added another label for PRL as the “hormone of parenthood” (Schradin & Anzenberger, 1999). Although PRL plays an important role in parental care, it is not the only factor which induces caregiving behaviour for the young. This complex physiological process normally involves a number of factors to ensure its proper function especially in higher vertebrates. Hence, steroid hormones including estradiol and progesterone are important for the proper functions of PRL (Schradin & Anzenberger, 1999).

2.3.2.1 Parental care in mammals

In almost all mammals, maternal care is long well known and females play vital role in parental care including the two families of monotremes, the platypus (Ornithorhynchidae) and the spiny anteater (Tachyglossidae) which bear egg (Bole-Feysot *et al*., 1998; Freeman *et al*., 2000; Reynolds *et al*., 2002). PRL and PL are suggested to induce maternal care because there is a significant increase in the circulating concentration of these hormones during and after the pregnancy (Bridges *et al*., 1990, 1997). PRL remains the sole factor in milk production in the mammary glands even though the stimulation and regulation of milk production may vary among vertebrates (Bole-Feysot *et al*., 1998; Neville *et al*., 2002).

In terms of paternal care, males are suggested to contribute care to the young. However, this is not always the case for all the mammals. Only a small portion in mammals is socially monogamous. However, monogamy isn't always equivalent to parental care (Schradin & Anzenberger, 1999; Freeman *et al*., 2000). Fathers showed a significant increase in plasma PRL in California mouse (*Peromyscus californicus*) and exhibited caregiving to the young (Gubernick *et al*., 1993). Among carnivores, wolves showed biparental care where both parents participate in caregiving by regurgitation of food, licking and defending the pups. For higher vertebrates, not only mothers, fathers also showed significant higher level of plasma PRL if compared with nonfathers in primates and human (Schradin & Anzenberger, 1999). Even though there is a connection between PRL and parental behaviour, in higher animals, the concentration of PRL may not regulate the parental behaviour of the animal (Schradin & Anzenberger, 1999). Across the mammalian taxa, only 9% of males help to provide care for the young including feeding, guarding, grooming, carrying, defending, teaching and play with the young offspring (Reynolds *et al*., 2002).

2.3.2.2 Parental care in birds

All birds showed biparental care behaviour, which is to say, both parents participate in taking care of their offspring (Reynolds *et al*., 2002). Studies showed that avian PRL

involves in incubation and brooding processes, including nesting behaviour and nest attendance (Hector & Goldsmith, 1985; March *et al*., 1994). Even though PRL is essential in incubation in doves, but other factors like gonadal hormones must also be taken into consideration (Janik & Buntin, 1985).

PRL secretion increased before hatching, especially during incubation period and it controls the parental behaviour (Buntin *et al*., 1996) and elevation of plasma PRL has been observed before incubation period in domestic chicken (Hall *et al*., 1986). This lends further support regarding the involvement of PRL in pre-hatching phase. Extensive research has been done mainly on ring dove regarding PRL and parental care. Results showed that injection of PRL made both parents show extensive care towards the young (Schradin & Anzenberger, 1999).

In birds, PRL induces proliferation and thickening of mucosal epithelial lining of pigeons and doves crop sac directly and indirectly, but not in every bird (Anderson *et al*., 1984). This crop sac is connected to the stomach and serves as a food storage organ. PRL acts as a mitogen on the epithelium and sloughs it off into the crop in order to produce a substance to nourish their young, called crop milk, similar function of mammary secretion (Anderson *et al*., 1984; Horseman & Buntin, 1995; Buntin *et al*., 1996). Crop milk contains clumps of epithelial cells from the crop sac mucosal lining wall and is fed to the young by regurgitation. PRL is proven to stimulate regurgitation feeding in birds (Horseman & Buntin, 1995; Buntin *et al*., 1991).

Regulation of PRL release in birds is controlled by a PRL-releasing factor, PRF, of the hypothalamus. Electrochemical (EC) stimulation of the preoptic-anterior hypothalamic region showed an increase in the weight of the crop sac of the pigeon. And studies showed that PRL strongly stimulates feeding activity and body weight gain in ring dove (Kanematsu, 1980). Other than stimulates crop sac proliferation, PRL synergizes with ovarian steroids to maintain the brood patch in many bird species (Bole-Feysot *et al*., 1998).

2.3.2.3 Parental Care in fish

Various studies have shown that PRL induced parental fanning to provide a constant supply of fresh water to the eggs in several teleosts (Slijkhuis *et al*., 1984; De Ruiter *et al*., 1986; Páll *et al*., 2004). In stickleback, PRL cells of the anterior pituitary were found to be more active during the later parental phase than during the initial sexual phase. Results suggested that this upsurge of PRL induces fanning but reduces courtship behaviour in stickleback (De Ruiter *et al*., 1986; Páll *et al*., 2004).

Besides, PRL induced nest building (Kindler *et al*., 1991) and also stimulated mucus production on body surface as a supplementary nutrient to feed the young. PRL treatment to discus fish enhanced the production of mucus-secreting cells, which serves as nutrient for the fries. In many fish, mucus secretion is responsive to PRL. It is further proven that the production of a substance for the survival of the young is not a feature restricted only to mammals (Ogawa, 1970; Blüm & Fiedler, 1965).

PRL was also shown to calm fish and to depress feeding response in cichlid. Results suggested that this behaviour can prevent the eggs to be eaten up by the parents. Further studies on other hormones including progesterone and several adenophypophysial hormones showed that PRL remained the sole factor in promoting parental behaviour of cichlids (Blüm & Fiedler, 1965). In paradise fish (*Macropodus opercularis*), males exhibited better parental behaviour than the females. However, studies showed that PRL alone could not increase bubble nests building, ie. bubble nests were formed by mixing air bubbles with mucus during egg laying process. Furthermore, androgen is found to increase nest building with the presence of PRL (Machemer & Fiedler, 1965 in Bole-Feysot *et al*., 1998). PRL is also involved in migration of some teleosts from seawater to fresh water (Bole-Feysot *et al*., 1998).

2.4 Discus as an animal model

Discus fish (*Symphysodon aequifasciata*) is a well-known and popular freshwater ornamental fish produced in several countries in South East Asia. These living jewels are

widely bred throughout Asian countries such as Malaysia, Singapore, Thailand and Indonesia with several breeding techniques developed to boost the production. From just a few original forms of wild discus, there is a variety of strains exists due to active interbreeding among wild forms and also among new varieties (Koh *et al*., 1999).

Discus fish was classified in the family of Cichlidae and *Symphysodon discus* was the first discus fish found and described by Johann Jacob Heckel at the Amazon River basin in 1840 while Jacques Pellegrin described the second species, the green discus, *Symphysodon aequifasciatus* in 1904. Subsequently, there were five subspecies documented in the later years, which are the *S. discus discus, S. discus willischwartzi, S. aequifasciatus aequifasciatus, S. aequifasciatus axelrodi and S. aequifasciatus haraldi*. Unfortunately, the identification and classification of new varieties were getting tougher since there was no genetic background recorded for the new varieties, like newborns that drifted from the lineage of their ancestors. Nowadays, discus was classified based on the colour and the physical characteristic, either from the wild-type or the domestic-type (Leibel, 1996).

Despite all nomenclature issues, more and more varieties of discus were produced and progressively exported to developed countries such as Japan, United Kingdom and the United States. Malaysia is one of the top discus fish contributors in the world with successful discus fish breeders and active discus breeding in the country. Today, discus fish are priced from RM30 to several thousands per fish according to the strains and sizes.

Discus fish (*Symphysodon aequifasciata*) belongs to Cichlidae family. Like other cichlids, all *Symphysodon* species are laterally compressed and round in shape like a disc. This is how the common name, “discus” is derived. The sides of the fish body are patterned generally. The height and length of the grown fish are both about 20–25 cm (8–10 in). This fish demonstrates unique parental-care behaviour towards newly hatched fries where parental fish feed their newborns with epidermal-secreted mucus (Chong *et al.*, 2005). There are other members in this family that feed their newborns with secreted mucus such as tilapia. The male parental-fish tilapia “incubates” free swim larvae in the mouth where newborns were fed with mucus secreted from the mouth (Kishida and Specker, 2000).

Parental discus fish will look after the eggs until they hatch and are able to free swim. During the eggs guarding period, the male and female parental fish will take turn to fan the eggs (Leibel, 1996). This study demonstrated that one very important contribution from parental-care behaviour in fish is indirect transferring of antimicrobial substances from epidermal mucus to fertilized eggs during egg-guarding session. Parental fish will normally move hatched embryos to other places in the aquarium which is safer and well covered in order to minimize distractions. Parental-care behaviour will continue until the larvae free swim and detach from the yolk sac. When larvae free swim, they will feed on the mucus secreted on the epidermis of the parental fish. Figure 2.5 shows discus fish and their unique parental-care behaviour.

There are more than 1300 species in the family Cichlidae and they are widespread in Africa and Central and South America. All species provide parental care. The diversity of forms of parental care is unusually large for a single family of animals. A review found evidence that 73 genera provide biparental care, and 108 genera have female care (Goodwin *et al*., 1998). Cichlid fish provide the best opportunity for the study of parental care in teleosts because they show diverse forms of parental care from substrate guarding to delayed and immediate mouthbrooding. There are also variations in which sex provides care (biparental, female-only and male-only care) (Blumer, 1982). Moreover, extensive ecological, behavioural and phylogenetic information is available for this family, which has led to considerable speculation about the studies of the uniqueness of fish parental care.